IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 9880
Shunji Natsuka, et al.	Examiner: Taeyoon Kim
Application No.: 10/700,505	Technology Center/Art Unit: 1651
Filed: November 5, 2003	
For: MURINE ALPHA (1,3) FUCOSYLTRANSFERASE FUC-TVII, DNA ENCODING THE SAME, METHOD FOR PREPARING THE SAME, ANTIBODIES RECOGNIZING THE SAME, IMMUNOASSAYS FOR DETECTING THE SAME, PLASMIDS CONTAINING SUCH DNA, AN CELLS CONTAINING SUCH PLASMID	**DECLARATION UNDER 37 C.F.R. § 1.131**
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. We, John B. Lowe, Kevin M. Gersten and Shunji Natsuka, were at the time of the invention employed by the Howard Hughes Medical Institute and the Regents of the University of Michigan, the assignee of the above-referenced patent application. Specifically, at the time of the invention, John B. Lowe was employed by the Howard Hughes Medical Institute and a faculty member of the University of Michigan. Dr. Lowe was a Howard Hughes Medical Institute Investigator of his own laboratory and supervisor to Kevin M. Gersten and Shunji Natsuka. Kevin M. Gersten was employed by the University of Michigan and a graduate student in Dr. Lowe's laboratory. Shunji Natsuka was employed by the Howard Hughes Medical Institute and a postdoctoral fellow in Dr. Lowe's laboratory. We are, the named and true inventors of the subject matter disclosed and claimed in the above-referenced patent application.

2. The present invention provides a murine fucosyltransferase-VII ("Fuc-TVII") enzyme comprising a catalytic domain that is encoded by a nucleic acid sequence segment that is identical to a polynucleotide that is amplified using murine mRNA or cDNA as a template by a 5' primer as shown in SEQ ID NO:3 (GCGCGGATCCCACCATCCTTATCTGGCACTGGCCTTTCACC) and a 3' primer as shown in SEQ ID NO:4 (GCGCGGATCCAGTTCAAGCCTGGAACCAGCTTTCAA GGTCTTC).

3. We conceived of and reduced to practice the claimed invention in the United States prior to June 7, 1995, the filing date of U.S. Patent No. 5,858,752. The attached Exhibits A-I are pages from the notebooks of Dr. Gersten, Dr. Natsuka, and Dr. Robert Kelly. Dr. Kelly was at the time of the invention a technician in Dr. Lowe's laboratory who worked under the supervision of Dr. Lowe. The data in the notebook pages provide evidence of the conception of the invention and its reduction to practice before June 7, 1995. The data accompanying this Declaration, with dates redacted from all documents, are as follows:

Exhibit A includes order confirmations for primers corresponding to SEQ ID NO:3 and SEQ ID NO:4 prior to June 7, 1995.

Exhibit B shows a laboratory notebook entry describing the successful cloning of murine FucT-VII stem region and catalytic domains from phage 104 using primer "624B" and "625B."

Exhibit C provides copies of orders of primers used to sequence the mouse FucT-VII gene from phage 104.

Exhibit D provides a list of the primers presented in Exhibit C in the order of their appearance (5' to 3') along the length of the mouse FucT-VII gene.

Exhibit E provides the full-length nucleic acid sequence of the mouse FucT-VII gene, as sequenced from phage 104, annotated with the sequence identification numbers of the primers, as listed in Exhibit D.

Exhibit F provides a notebook page from co-inventor Shunji Natsuka, wherein Dr. Natsuka correlates phage 104 with mouse FucT-VII.

Exhibit G provides pages from the laboratory notebook of Dr. Robert Kelly discussing the transfection of COS-7 cells with expression vectors containing nucleic acids encoding different mouse FucT-VII sequences. Transfection of the COS-7 cells is confirmed by chloramphenicol acetyl transferase (CAT) and protein concentration assays.

Exhibit H provides flow cytometry profiles of the COS-7 cells transfected with expression vectors containing nucleic acids encoding different mouse FucT-VII sequences and contacted with antibodies to detect α-1,3-fucosylated glycan epitopes including sialyl Lewis X. Enzymatic activity of the FucT-VII proteins is confirmed by flow cytometry analyses.

Exhibit I provides pages from the laboratory notebook of Dr. Robert Kelly discussing *in vitro* FucT-VII assays using detergent extracts of COS-7 cells transfected with expression vectors containing nucleic acids encoding different mouse FucT-VII sequences.

4. Page 1 of **Exhibit A** shows primer "624B," which is a forward (sense) primer that is identical to SEQ ID NO:3. Page 2 of Exhibit A shows primer "625B," which is a reverse (antisense) primer that corresponds to SEQ ID NO:4. Primer 625B is different from SEQ ID NO:4 at the three nucleotide bases in between the BamHI restriction endonuclease sequence (underlined) and the stop codon (in bold). Because the below primers are antisense, the stop codon read in the sense orientation is TGA (opal stop codon).

SEQ ID NO:4: GCGCGGATCCAGT**TCA**AGCCTGGAACCAGCTTTCAAGGTCTTC

625B: GCGCGGATCCTCA**TCA**AGCCTGGAACCAGCTTTCAAGGTCTTC

5. SEQ ID NO:4 and primer 625B anneal to the identical coding sequence of murine Fuc-TVII. Primer pairs composed of SEQ ID NO:3 and SEQ ID NO:4 or 624B and 625B will

amplify the same stem region and catalytic domain sequence from a murine Fuc-TVII nucleic acid template.

6. **Exhibit B** describes how primers 624B and 625B were used to amplify the stem and catalytic domains of murine Fuc-TVII. The stem and catalytic domain of murine Fuc-TVII were amplified from phage 104, which contains the murine Fuc-TVII gene. Exhibit B discusses specific PCR conditions and shows agarose gels of pET3b cloning plasmids with the cloned murine Fuc-TVII sequence excised by a BamHI restriction endonuclease treatment.

7. **Exhibit C** provides copies of orders of primers used to sequence the mouse FucT-VII gene from phage 104. The primers were ordered by co-inventor Kevin M. Gersten for the purpose of sequencing phage 104. In the "User Comments" section of the orders, Kevin M. Gersten interchangeably refers to FucT-VII or phage 104. The primer sequence number or system identification number is indicated in the upper right hand corner of the orders (*e.g.*, "Sequence # 3242B"). The primers, named according to their sequence number or system identification number, are listed in the order of their appearance (5′ to 3′) along the length of the mouse FucT-VII gene in **Exhibit D**.

8. **Exhibit E** provides the full-length nucleic acid sequence of the mouse FucT-VII gene, as sequenced from phage 104, annotated with the sequence identification numbers of the primers used by Kevin M. Gersten. The primer sequences are identified by bolded text. The primer sequence identification number is above the sequence for forward primers and below the sequence for reverse primers. Start and stop codons and relevant restriction endonuclease sites are also identified.

9. **Exhibit F** shows a notebook page from co-inventor Shunji Natsuka, who used a segment of the mouse FucT-VII gene in phage 104 to clone the human FucT-VII gene. On the notebook page presented, Dr. Natsuka records using a fragment from the FucT-VII gene in phage 104 to hybridize to human genomic DNA in a Southern blot. At the top of the page, Dr. Natsuka correlates phage 104 with mouse FucT-VII.

10. **Exhibit G** shows transfection efficiency analysis of COS-7 cells transfected with expression vectors encoding different mouse FucT-VII constructs, along with a negative control vector. The constructs cDNA 3, 10 and 14 correspond to Figure 1a; page 8, line 7 through page 9, line 25; and page 62, line 14 through page 67, line 2 of the present application. These data are also published in Smith, *et al.*, *J Biol Chem* (1996) 271(14):8250-8259 (*see*, *e.g.*, Figure 1). The constructs "FT7 1,2a2b,3;" "FT7 1,2b,3;" "FT7 1,3" refer to artificial constructs assembled from identified exons from the mouse FucT-VII gene (*see*, Figure 1a of the present application and Figure 1 of Smith, *et al.*). Each cDNA and artificial construct also includes exon 4 of the FucT-VII gene, as depicted in Figure 1a of the present application and Figure 1 of Smith, *et al.*, and therefore contain the stem and catalytic domains amplified using the primers SEQ ID NO:3 and SEQ ID NO:4

11. The COS-7 cells transfected with FucT-VII constructs were subjected to three types of analyses:

a. CAT enzymatic activity assays, to allow quantification of transfection efficiency;
b. protein concentration assays, to allow quantification of the relative "specific activity" for the CAT assays, the results of which confirmed that the cells were transfected with the FucT-VII cDNA expression vectors; and
c. flow cytometry confirming enzymatic activity of the expressed constructs, using antibodies against cell surface α-1,3-fucosylated glycans that are potential products of FucT-VII enzymatic activity.

12. The notebook pages of **Exhibit G** record experiments in which various cDNAs corresponding to transcripts from the mouse FucT-VII locus were cloned into a mammalian expression vector called pcDNA1, yielding *e.g.*, cDNAs 3, 10, 14 and an artificial construct assembled from exons 1, 2a, 2b, 3 and 4 from FucT-VII ("FT7 1,2a2b,3"). The resulting expression vectors containing FucT-VII constructs were transfected into COS-7 cells, along with a co-transfected expression vector encoding chloramphenicol acetyl transferase (CAT). The specific activity of the CAT assays was confirmed by protein concentration assays. The results

depicted on page 067 of **Exhibit G** confirm that the COS-7 cells were successfully transfected with each of the FucT-VII cDNA expression vectors, on the basis of CAT activity normalized to protein concentration.

13. **Exhibit H** shows the profiles of flow cytometry analyses to confirm enzymatic activity of the expressed FucT-VII cDNAs. The flow cytometry was performed using antibodies against the following cell surface α-1,3-fucosylated glycan epitopes, labeled at the top of the flow histograms:

i. H - a negative control antibody that would recognize α-1,2-fucosylated glycans; Samples 001-007.
ii. LX - Lewis x; Samples 008-014
iii. SLX - Sialyl Lewis X Samples 015-021
iv. LA - Lewis a Samples 022-028
v. SLA - Sialyl Lewis a Samples 028-035

14. The data from **Exhibit H** show that mouse FucT-VII expression vectors corresponding to cDNA10, cDNA14 (as indicated in Figure 1a), and FT7 1,2a2b,3 direct expression of the sialyl Lewis X epitope on COS-7 cells (*i.e.*, Samples 016, 020, 021), but not the three other possible α-1,3-fucosylated glycan epitopes that α-1,3-fucosyltransferases are known to synthesize. This pattern of cell surface glycan antigen expression is characteristic of and idiosyncratic to FucT-VII, as published in Smith, *et al.*, *J Biol Chem* (1996) 271(14):8250-8259, and demonstrates that the cells transfected with the expression vectors corresponding to cDNA10, cDNA14, and FT7 1,2a,3 thus express mouse Fuc-TVII. The expression vector and antibody used are at the top left, where it says "Sample." The relevant data are in each profile, which show graphically the number of cells that are to the right of the vertical line which is set in the negative control

experiments to include more than 98% of the cells to the left of the line. Cells to the right of the line are "positive" for the cell surface antigen being analyzed. This line thus discriminates between cells that are "positive" for the glycoantigen, and "negative" for the glycoantigen. These data are quantified at the bottom of each profile. The % of all cells to the right of the vertical line that discriminates between positive and negative is shown on the second line of numbers under the column labeled "% Gated". For example, in the profiles of Samples 016, 020 and 021, testing respectively FT7 1,2a2b,3, cDNA10 and cDNA14 transfected cells stained with anti-Sialyl Lewis X (SLX), 23.6%, 14.9% and 8.0%, respectively, of the cells are "positive" for the Sialyl Lewis X antigen. The results of the flow cytometry analyses confirm the expression and enzymatic activity of the FucT-VII proteins expressed from the expression vectors transfected into the COS-7 cells.

15. The data from **Exhibit I** show *in vitro* enzyme assays using detergent extracts made from COS-7 cells transfected with an expression vector containing the artificial construct FT7 1,2a2b,3 (also, "mFT7"). This experiment was done to generate the radioactive Sialyl Lewis X moiety, the primary mouse FucT-VII product. The data of Exhibit I confirm that the mFT7 expression vector programs COS-7 cells to contain fucosyltransferase enzyme activity that synthesizes the Sialyl Lewis X tetrasaccharide. Enzyme activity was still observed when the expressed recombinant FT7 1,2a2b,3 was isolated in detergent extracts.

16. In this experiment recorded in **Exhibit I**, COS-7 cells were transfected with the mouse FT7 1,2a2b,3 vector, or with a positive control vector encoding human FucT-III (labeled hFT3). The transfected cells were lysed in 1% Triton X-100, and the resulting soluble extracts containing the recombinantly expressed fucosyltransferases were used in an *in vitro* fucosyltransferase enzyme assay. These assays are done in an enzyme "cocktail" containing various constituents, including the fucosyltransferase donor substrate GDP-fucose where the fucose is radioactive with carbon 14. The assays were done in the presence or absence of the fucosyltransferase acceptor substrate sialyl N-acetyllactosamine (labeled SLN), a trisaccharide. Assays without acceptor are labeled "-acceptor" or "-Acc". Assays with acceptor are labeled

"+acceptor" or "+Acc". If the enzyme was active with the acceptor substrate, the acceptor substrate was enzymatically modified by the radioactive fucose to form the product, in this case Sialyl Lewis X. After the enzyme reaction was complete, the entire cocktail was processed by ion exchange chromatography which retained the radioactive GDP-fucose on the column, allowing any radioactive product (Sialyl Lewis X) and non-product radioactivity also present in the reaction (*e.g.*, radioactive impurities of undefined nature in the GDP-fucose) to flow through the column and be counted in a scintillation counter. The non-product radioactivity was assessed by doing an enzyme reaction in the absence of acceptor substrate. The specific enzyme activity was quantified by measuring the radioactivity incorporated into the acceptor substrate in the "+Acc" reaction, and subtracting the radioactivity that was present in the "-Acc" reaction. The data are presented on page 097 on the scintillation counter printout. The first three rows are the "+Acc" assays, using extracts, one of which is FT7 1,2a2b,3. The second three rows are the "-Acc" assays, which are the non-product radioactive impurities. For FT7 1,2a2b,3, there were 1651.20 counts eluted from the "+Acc" column (position 003) and 679.70 counts eluted from the "- Acc" column (position 006), demonstrating a specific activity of 971.50 counts, corresponding to the mouse FucT-VII product Sialyl Lewis X, for FT7 1,2a2b,3 in isolated cell membrane detergent extracts.

17. The above evidence demonstrates that prior to June 7, 1995, we had conceived of and reduced to practice the claimed invention. As shown in Exhibit A, we had designed primers corresponding to SEQ ID NO:3 and SEQ ID NO:4 specifically for the amplification of murine Fuc-TVII stem region and catalytic domain from a murine Fuc-TVII gene template. As shown in Exhibit B, we further used the primers to amplify and clone the murine Fuc-TVII stem region and catalytic domain. Exhibits C-F unequivocally establish that phage 104 contained the FucT-VII gene. Exhibits G-I confirm the recombinant expression and enzymatic activity of FucT-VII enzymes recombinantly expressed from several different mouse FucT-VII constructs containing exon 4, which encodes the stem and catalytic regions. We observed enzymatic activity from our recombinantly expressed FucT-VII proteins regardless of whether the enzyme was in an intact cell or isolated in cell membrane detergent extracts.

18. In view of the foregoing, we respectfully submit that the evidence provided in Exhibits A through I unequivocally establishes that the claimed invention was conceived of and reduced to practice prior to June 7, 1995.

19. We further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

20. The Declarants have nothing further to say.

Dated: 4-22-08

John B. Lowe

Dated:_____

Kevin M. Gersten

Dated:_____

Shunji Natsuka

Attachments
JLW:jlw
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